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Innate and adaptive immune control of genetically engineered live-attenuated arenavirus vaccine prototypes

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Abstract

Arenaviruses such as Lassa virus (LASV) cause significant morbidity and mortality in endemic areas. Using a glycoprotein (GP) exchange strategy, we have recently developed live-attenuated arenavirus vaccine prototypes (rLCMV/VSVG) based on lymphocytic choriomeningitis virus (LCMV), a close relative of LASV. rLCMV/VSVG induced long-term $CD8^+$ T cell immunity against wild-type virus challenge and exhibited a stably attenuated phenotype *in vivo*. Here we elucidated the innate and adaptive immune requirements for the control of rLCMV/VSVG. Infection of $RAG^{-/-}$ mice resulted in persisting viral RNA in blood but not in overt viremia. The latter was only found in mice lacking both RAG and IFN type I receptor. Conversely, absence of IFN type II signaling or NK cells on an RAG-deficient background had only minor effects on vaccine virus load or none at all. rLCMV/VSVG infection of wild-type mice induced less type I IFN than did wild-type LCMV, and type I as well as type II IFNs were dispensable for the induction of virus-specific memory CD8 T cells and virus-neutralizing antibodies by rLCMV/VSVG. In conclusion, the adaptive immune systems are essential for elimination of rLCMV/VSVG, and type I but not type II IFN plays a major contributive role in lowering rLCMV/VSVG loads *in vivo*, attesting to the attenuation profile of the vaccine. Nevertheless, IFNs are not required for the induction of potent vaccine responses. These results provide a better understanding of the immunobiology of rLCMV/VSVG and will contribute to the further development of GP exchange vaccines for combating arenaviral hemorrhagic fevers.

Keywords: arenavirus, Lassa fever, live attenuation, lymphocytic choriomeningitis virus, vaccine

Introduction

Smallpox has been eradicated by vaccination and similar attempts for poliovirus hold promise, while the fight against other viral pathogens is far less advanced and remains an urgent biomedical challenge (1). The Old World arenavirus Lassa virus (LASV) can cause hemorrhagic fever in humans and falls into the latter category of pathogens. In the endemic areas of West Africa, it causes an estimated number of 300 000 clinically manifest infections each year, leading to a few thousand deaths (2). Furthermore, LASV and other hemorrhagic arenaviruses are a concern owing to their potential misuse in biological warfare (3). Still, the available options for treatment are unsatisfactory and—with the exception of Junin virus (4)—vaccines remain unavailable for clinical use. Several strategies are being pursued for creating an LASV vaccine, including DNA vaccination and bacterial as well as viral vectors. However, live-attenuated vaccines are often the most efficient in providing protection against infection particularly when aiming for long-term immunity (5–7). Mopeia virus—a close relative of LASV—as well as reassortant viruses containing Mopeia and LASV

gene segments have therefore been considered as LASV vaccine candidates (8–12). Still, there remains a need for vaccine candidates with an optimal efficacy and safety profile. By exploiting the reverse genetic system for lymphocytic choriomeningitis virus (LCMV) (13, 14), we have recently shown that live-attenuated arenavirus vaccine prototypes can be generated by exchange of their glycoprotein (GP) for the unrelated GP of vesicular stomatitis virus (VSV) (15), a rhabdovirus. The resulting vaccine prototypes (rLCMV/VSVG) were attenuated in cell culture and in mice; yet, they were still able to induce vigorous CD8 T cell responses against virus internal proteins and to confer long-term protection against wild-type LCMV challenge (15). In addition, potent antibody responses to the foreign VSVG envelope protein were elicited and protected against lethal VSV challenge (16).

We have previously demonstrated that rLCMV/VSVG has lost the pathogenic potential of wild-type LCMV (15–17). However, this vaccine prototype is replication competent and has hence raised a number of questions related to its potential future use in immunocompromized patients. Accordingly, the high prevalence of HIV in LASV endemic areas has been a major impediment to the introduction of recombinant vaccinia virus-based LASV vaccines. It was therefore of interest to analyze mechanisms of rLCMV/VSVG immune control *in vivo* and to investigate immune responses in animal models of immunodeficiency. Here we have dissected the role of individual innate and adaptive immune pathways in rLCMV/VSVG control and elimination. For this purpose, we exploited a range of gene-targeted mouse models (described in Table 1) that lack type I and type II IFN signaling, T and B cells (owing to deficiency in recombination activation gene 1) and NK cells in various combinations. Since the role of innate immunity in facilitating vaccination has become increasingly appreciated, we have aimed to characterize the dependence of rLCMV/VSVG-induced vaccine responses on IFN signaling, since the role (18–21).

In this study, we found that adaptive immunity is key for eliminating our live-attenuated arenavirus vaccine prototype. Furthermore, type I IFN contributed substantially to keep the infection in check, in a manner independent of T and B cells, thereby determining systemic vaccine virus loads in animals lacking adaptive immunity. This study also revealed that rLCMV/VSVG induced less type I IFN than wild-type LCMV (LCMV-ARM) and that type I and II IFNs are both dispensable for the induction of potent vaccine-induced cellular and humoral immune responses.

Methods

Mice and animal experiments

IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ (22), RAG$^{-/-}$ (23), RAG$^{-/-}$ γc$^{-/-}$ (24), RAG$^{-/-}$ IFNα/βR$^{-/-}$ (25), RAG$^{-/-}$ IFNγR$^{-/-}$ (25) and RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ (25) (see also Table 1) and C57BL/6 as well as 129Sv/Ev wild-type control mice were bred and housed under SPF conditions at the Institute of Laboratory Animal Science, University of Zurich. Animal experiments were carried out with authorization of the Veterinäramt of the Kanton Zürich and in accordance with the Swiss law for animal protection.

Virus strains, immunofocus assay and infection protocol

LCMVARM5.3b (LCMV-ARM) is a triple plaque-purified isolate of ARM CA 1371 originally obtained from M. J. Buchmeier (The Scripps Research Institute, La Jolla, CA). rLCMV/INDG and rLCMV/NJG were generated by reverse genetic techniques in which the LCMV GP ORF was substituted with either the ORF of VSV G serotype Indiana (IND) or serotype New Jersey (NJ) (compare Supplementary Figure 1A, available at *International Immunology* Online, and 15, 17). The two recombinant viruses as a common entity are also referred to as 'rLCMV/VSVG'. All viruses were grown on BHK-21 cells and viral infectivity was titrated in a standard immunofocus assay on MC57G cells (27). The mice were infected intravenously at a standard dose of $\sim 2 \times 10^5$ PFU unless stated differently.

IFNα ELISA

Concentration of mouse IFNα in 1:10 with PBS prediluted sera was measured by ELISA according to the manufacturer's suggestions (PBL Interferon Source, Piscataway, NJ, USA).

Virus-specific CD8$^+$ T cell quantification and VSV neutralization assay

CD8$^+$ T cell responses were measured by flow cytometry using H2-D^b tetramers loaded with the LCMV-derived peptide epitope NP396–404 combined with antibodies specific to the surface markers CD8 and B220/CD45R (BD Pharmingen,

Table 1. Gene-deficient mouse models used in this study

Knockout mouse strain description	Background[a]	T cells	B cells	NK cells	IFNα/β R	IFNγ R	References
IFNα/βR$^{-/-}$ IFNγR$^{-/-}$	129Sv/Ev	+	+	(+)[b]	–	–	(22)
RAG$^{-/-}$	C57BL/6	–	–	+	+	+	(23)
RAG$^{-/-}$ γc$^{-/-}$	C57BL/6	–	–	–	+	+	(24)
RAG$^{-/-}$ IFNα/βR$^{-/-}$	129Sv/Ev/C57BL/6 mixed	–	–	(+)[b]	–	+	(25)
RAG$^{-/-}$ IFNγR$^{-/-}$	129Sv/Ev/C57BL/6 mixed	–	–	(+)[b]	+	–	(25)
RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$	129Sv/Ev/C57BL/6 mixed	–	–	(+)[b]	–	–	(25)

[a]129Sv/Ev and C57BL/6 mice are equally resistant to both, wild-type LCMV (26) and rLCMV/VSVG (data not shown), justifying the comparative analysis of the different knockout strains on C57BL/6 and C57BL/6 x 129Sv/Ev mixed backgrounds.
[b]Mice deficient in IFN I receptor, IFN II receptor or both have been reported to have immature NK cells with severely impaired cytotoxic activity (30–32).

Allschwil, Switzerland) (15). The frequencies shown represent the percentage of tetramer-positive cells within $CD8^+$ $B220^-$ peripheral blood lymphocytes. VSV-neutralizing antibody titers were determined in a plaque reduction assay on Vero cells as previously described (16).

RNA isolation and quantitative reverse transcription–PCR for the detection of LCMV nucleoprotein

Viral RNA was isolated from mouse serum using the QIAamp Viral RNA Mini kit (Qiagen, Hombrechtikon, Switzerland). The following oligonucleotides specific to the nucleoprotein (NP) gene of LCMV strain ARM were designed with the software Primer Express version 1.5 (Applied Biosystems, Rotkreuz, Switzerland) to generate a 75-nt-long amplicon: forward primer NP1992f: 5′-ACTGACGAGGTCAACCCGG-3′, reverse primer NP2057r: 5′-CAAGTACTCACACGGCATGGA-3′ and TaqMan probe: NP2035: 5′-FAM-CTTGCCGACCTCTTCAATGCGCAA-BHQ1-3′. Assay oligonucleotides and details have been deposited in the public database RTPrimerDB with ID number 7815.

For absolute quantification of LCMV RNA copies, a 401-nt-long PCR product was amplified using the primers NP2206r-T7overhang 5′-gaaattaatacgactcactataggGAGGAATTGACCCCAACGCT-3′ (small letters indicate T7 promoter and upstream sequence) and NP1808f 5′-TCCAzGTATGCATCTTACACAACCAG-3′ on a plasmid template containing the NP ORF of LCMV-ARM. The amplicon was gel purified with the QIAquick Gel Extraction kit (Qiagen) and subsequently used to generate RNA standards by T7 *in vitro* transcription. After DNase digestion with the Turbo DNA-free kit (Ambion, Rotkreuz, Switzerland), *in vitro* transcripts were quantified by RNA 6000 Nano RNA Chip on a 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland) to calculate the number of RNA copies. This synthetic RNA was serially 10-fold diluted with DEPC-treated H_2O containing 100 ng μl^{-1} yeast tRNA carrier (Ambion) in DNA LoBind tubes (Eppendorf, Schönenbuch, Switzerland) and used for standardization of the TaqMan assay. The following one-step quantitative reverse transcription–PCR (qRT-PCR) protocol was run using the TaqMan One-Step RT-PCR Master Mix (Applied Biosystems) and primers/probe at a concentration of 300 nM/250 nM: 30 min at 48°C; 10 min at 95°C; 45 cycles of 15 s at 95°C and 1 min at 56°C. Small amounts of an internal positive control were used to control for RNA extraction efficiency and downstream processing. This *in vitro* RNA transcript with binding sites for NP1992f and NP2057r but with a different probe-binding area was spiked into each collected serum sample prior to RNA isolation and was simultaneously quantified with a second probe labeled with a different fluorescent dye (5′-JOE-AAAGGCCAAGAAGGGCGGAAAGTCC-BHQ1-3′). All samples were run in duplicates on 7700SDS or 7900HTS machines (Applied Biosystems) and analyzed with the SDS1.9 or SDS2.0 software (Applied Biosystems).

Statistical analysis

One-way analysis of variance was used for the comparison of multiple groups, and *t*-tests (unpaired, two tailed; with Bonferroni correction for multiple comparisons) assessed differences between two groups (GraphPad Prism software 4.0a). Viral titers were log transformed for analysis. *P*-values >0.05 were considered not statistically significant, $P < 0.05$ was considered significant (*) and $P < 0.01$ as highly significant (**).

Results

Contribution of innate and adaptive immune responses to rLCMV/INDG control

First, we investigated the role of adaptive immunity in controlling rLCMV/VSVG. $RAG^{-/-}$ mice lack T and B cells and are known to develop lifelong overt viremia when infected with LCMV (LCMV-ARM, Fig. 1A) (28). In contrast, rLCMV/INDG or rLCMV/NJG infection failed to cause overt viremia (Fig. 1A). Still, viral RNA persisted in the blood of these animals (a status referred to as 'RNemia') but was only detectable when assessed with a highly sensitive qRT-PCR assay (Fig. 1B, Supplementary Figure 1 is available at *International Immunology* Online). These observations in $RAG^{-/-}$ mice indicated that adaptive immunity was necessary to eliminate rLCMV/VSVG, but they suggested also that innate immune pathways suppressed replication of this vaccine virus to fairly low levels.

IFNs play a key role in the control of many viruses including wild-type LCMV (22, 29). Thus, we hypothesized that IFNs might be at the root of strongly suppressed rLCMV/VSVG replication in $RAG^{-/-}$ mice, and we tested vaccine virus control in $IFN\alpha/\beta R^{-/-}$ $IFN\gamma R^{-/-}$ double-deficient animals. These mice are unable to control wild-type LCMV (22, 29) but they rapidly eliminated rLCMV/VSVG from blood (Fig. 1C and D). Two different explanations could have accounted for these results: (i) either IFN type I and II signaling was not involved in rLCMV/VSVG control or (ii) adaptive immune defense and IFNs exhibited considerable redundancy in controlling the vaccine virus. To discriminate between these possibilities, we analyzed rLCMV/VSVG control in mice lacking RAG as well as the IFN type I receptor ($RAG^{-/-}$ $IFN\alpha/\beta R^{-/-}$) or additionally lacking the IFN type II IFN receptor ($RAG^{-/-}$ $IFN\alpha/\beta R^{-/-}$ $IFN\gamma R^{-/-}$). Both genotypes of mice developed overt viremia, which was also reflected in RNemia levels exceeding those of $RAG^{-/-}$ mice. This suggested that IFN type I signaling potently contained rLCMV/VSVG infection. Thus, IFN type I signaling determined largely the level of viral persistence in the absence of adaptive immunity. In addition, small but reproducible differences in virus loads between $RAG^{-/-}$ $IFN\alpha/\beta R^{-/-}$ and $RAG^{-/-}$ $IFN\alpha/\beta R^{-/-}$ $IFN\gamma R^{-/-}$ mice ($P < 0.01$) pointed toward a minor contributive role of IFN type II signaling in vaccine virus control. Conversely, we could not detect a clear contribution of NK cells in controlling rLCMV/VSVG when testing its replication in $RAG^{-/-}$ mice additionally lacking the common γ chain of the IL receptors ($RAG^{-/-}$ $\gamma c^{-/-}$). $RAG^{-/-}$ $\gamma c^{-/-}$ mice are NK cell deficient but did not exhibit viremia and RNemia was in similar ranges as in $RAG^{-/-}$ mice. In line with this observation, combined IFN type I and type II receptor deficiency also results in impaired NK cell activity (30–32) but did not prevent efficient control of rLCMV/VSVG in $IFN\alpha/\beta R^{-/-}$ $IFN\gamma R^{-/-}$ mice (Fig. 1C and D).

Of note, neither the overt viremia in $RAG^{-/-}$ $IFN\alpha/\beta R^{-/-}$ and $RAG^{-/-}$ $IFN\alpha/\beta R^{-/-}$ $IFN\gamma R^{-/-}$ mice nor low-level RNemia

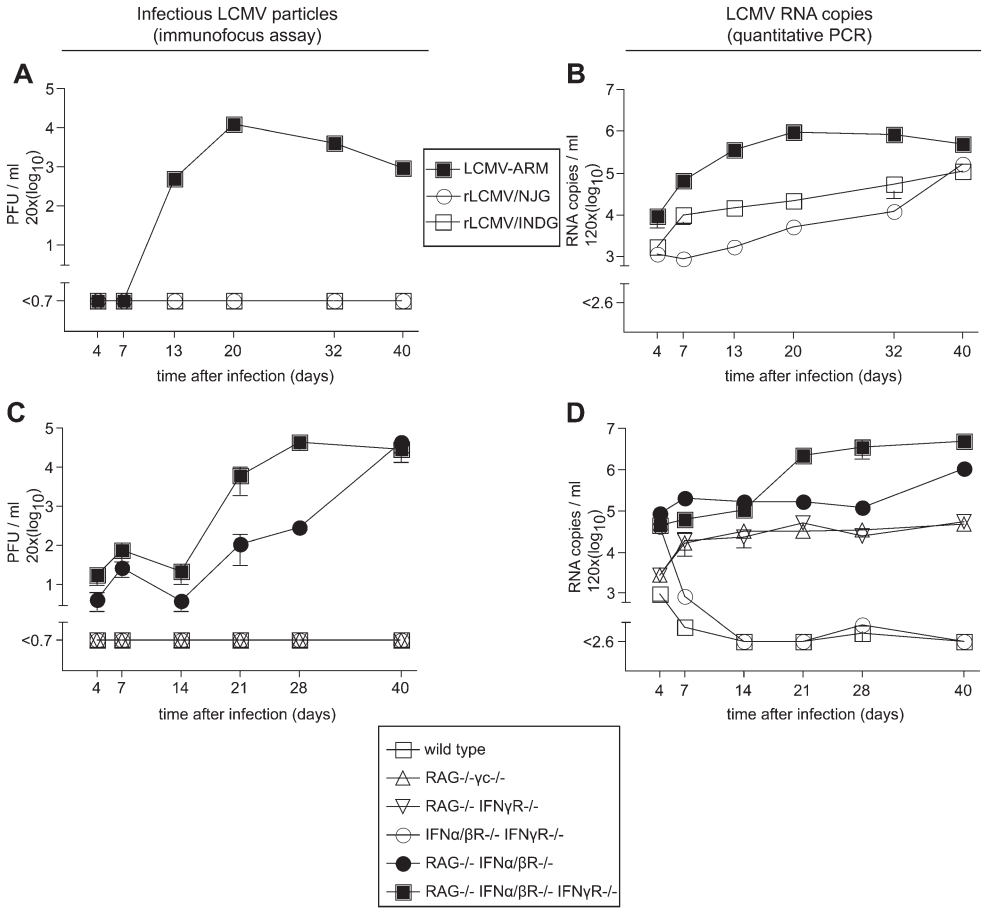

Fig. 1. Differential requirement for innate and adaptive immune responses in controlling rLCMV/INDG. (A and B): RAG$^{-/-}$ mice were infected with LCMV-ARM, rLCMV/INDG or rLCMV/NJG. Viral infectivity in blood was determined by immunofocus assay (A) and viral RNA copies in serum were quantified by qRT-PCR (B). (C and D): IFNα/βR$^{-/-}$ IFNγR$^{-/-}$, RAG$^{-/-}$ γc$^{-/-}$, RAG$^{-/-}$ IFNα/βR$^{-/-}$, RAG$^{-/-}$ IFNγR$^{-/-}$, RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice and wild-type mice were infected with rLCMV/IND and viral infectivity (C) and viral RNA copies (D) were determined by immunofocus assay and qRT-PCR, respectively. Symbols represent the mean ± standard error of the mean of four mice per group.

in the other RAG-deficient mouse strains was associated with manifestations of disease. This confirms our earlier observations on the apathogenic behavior of rLCMV/VSVG (15, 17, 33) and extends these findings to hosts with severe and combined immune deficiencies.

'RNemic' serum of RAG$^{-/-}$ γc$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice can transfer infection to RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ indicator mice

Persisting rLCMV/INDG in RAG$^{-/-}$, RAG$^{-/-}$ γc$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice could only be detected by qRT-PCR (Fig. 1). To test for residual infectivity, we transferred serum from rLCMV/INDG-infected RAG$^{-/-}$ γc$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice into highly susceptible RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice. This *in vivo* bioassay (schematically depicted in Fig. 2A) revealed residual low-level infectivity in the sera of six out of seven individual RAG$^{-/-}$ γc$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice, resulting in overt viremia of recipient RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice 30 days after serum transfer (Fig. 2B). Serum of viremic rLCMV/INDG-infected RAG$^{-/-}$ IFNα/βR$^{-/-}$ mice was also infectious as expected based on our immunofocus assay data (compare with Fig. 1C). In contrast, serum from IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice (free of detectable viral RNA) served as a negative control and failed to transfer infection to RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice, confirming that type I and II IFNs were dispensable for eliminating rLCMV/INDG infection in an RAG-competent background. This shows that rLCMV/INDG-infected RAG$^{-/-}$, RAG$^{-/-}$ γc$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice still harbored low levels of infectious virus, which was, however, below the detection level of the conventional immunofocus assay.

rLCMV/INDG elicits unimpaired CD8 T cell and antibody responses in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice

LCMV-ARM-induced T cell responses and antibody induction by VSV are known to greatly depend on type I IFN signaling (34–37). Nevertheless, elimination of rLCMV/VSVG in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice was apparently the result of adaptive immune responses and occurred independently of IFN type I signaling. Moreover, rLCMV/INDG and rLCMV/NJG induced significantly less serum IFNα than LCMV-ARM (Supplementary Figure 2 is available at *International Immunology* Online). These findings, together with unimpaired vaccine virus control

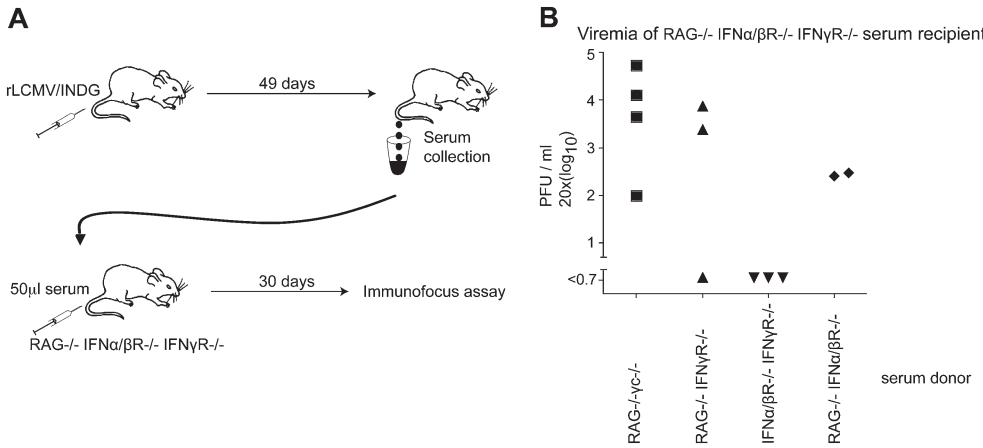

Fig. 2. Transfer of RNemic serum from aviremic mice is infectious for RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice. (A): Schematic description of the experimental setup. Sera were collected from individual RAG$^{-/-}$ γc$^{-/-}$, RAG$^{-/-}$ IFNα/βR$^{-/-}$, IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice infected 49 days previously with rLCMV/INDG and were transferred into individual RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ indicator mice. Thirty days after serum transfer, viral infectivity in the blood of the recipient RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice was measured by immunofocus assay (B). Viral titers of individual recipient indicator mice are shown.

in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice, raised the possibility that rLCMV/VSVG-induced T cell and antibody responses depended less on type I IFN than those induced by the parent wild-type viruses. To directly address this possibility, we studied the adaptive immune response to rLCMV/INDG in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice. CD8$^+$ T cells specific for the immunodominant LCMV-NP-derived epitope NP396–404 (NP396) were monitored in peripheral blood (Fig. 3A). Unlike reported from LCMV-ARM-infected IFN type I receptor-deficient mice (34–37), we found an unimpaired virus-specific CD8 T cell response in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice. It even reached 5- to 10-fold higher frequencies than in wild-type mice, and memory populations of specific CD8$^+$ T cells persisted throughout day 447 after immunization. To assess the functionality of these memory CD8$^+$ T cells, rLCMV/INDG immune splenocytes from IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice were transferred to naive syngeneic wild-type mice that were subsequently challenged with LCMV-ARM. After transfer, a small population of NP396-specific CD8$^+$ T cells was detected in the recipient mice (~0.2% 1 week after transfer, data not shown). Subsequent intracranial infection with LCMV-ARM led to a rapid expansion of these transferred memory CD8$^+$ T cells (Fig. 3B) and protected the recipients against lethal LCMV-induced choriomeningitis (n = 3, data not shown). Of note, protection in this challenge setting relies entirely on memory CD8 T cells (38), whereas B cell memory cannot contribute to protection owing to the different surface GPs of rLCMV/INDG (immunization) and LCMV-ARM (challenge). Hence, these results attested to the functionality of rLCMV/VSVG-induced memory CD8$^+$ T cells in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice. In analogy to CD8$^+$ T cell responses, rLCMV/INDG-specific neutralizing antibody responses in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice reached significantly higher peak titers than in wild-type mice and subsequently remained in the wild-type range for >6 months (Fig. 3C).

These results indicated that neither IFN type I nor IFN type II signaling were necessary for potent CD8 T cell responses and neutralizing antibody induction by rLCMV/VSVG and that memory formation was similarly unimpaired in the absence of these pathways.

Discussion

Vaccine design aims to provide optimal immunogenicity and protective capacity in combination with a reliable safety profile (39). Reverse genetic engineering allows the targeted attenuation of viruses as well as the directed enhancement of immunogenicity and incorporation of desirable immunomodulatory properties (1, 40). Here we found that rLCMV/VSVG, the first live-attenuated arenavirus vaccine prototype engineered by reverse genetic techniques, was largely controlled by type I IFN signaling but its elimination depended on adaptive immune responses. Accordingly, IFN type I signaling reduced viral replication in RAG$^{-/-}$, RAG$^{-/-}$ γc$^{-/-}$ and RAG$^{-/-}$ IFNγR$^{-/-}$ mice to below the detection limit of the immunofocus assay, whereas the combined deficiency in RAG and IFN type I receptor (i.e. RAG$^{-/-}$ IFNα/βR$^{-/-}$ and RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice) permitted viral replication at a level that was detectable as overt viremia. In addition, IFN type II had a moderate effect on rLCMV/VSVG replication when tested in the context of an IFN type I and RAG double deficiency (compare RAG$^{-/-}$ IFNα/βR$^{-/-}$ versus RAG$^{-/-}$ IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice in Fig. 3C and D).

We acknowledge that RAG-deficient mice exhibit a disrupted splenic microarchitecture with an incompletely developed marginal zone, a compartment which has been shown to contribute to type I IFN responses in LCMV infection (41). Thus, we cannot formally exclude the possibility that impaired IFN type I responses to rLCMV/VSVG in RAG-deficient animals may negatively affect innate immune control and thereby may further reduce these animals' ability to eliminate the vaccine virus. On the other hand, substantially different virus loads in RAG and RAG$^{-/-}$ IFNα/βR$^{-/-}$ mice demonstrate that the IFN type system is fairly capable to contain rLCMV/VSVG even in RAG-deficient mice. Thus, the efficiency of the type I IFN system in reducing rLCMV/VSVG replication may be

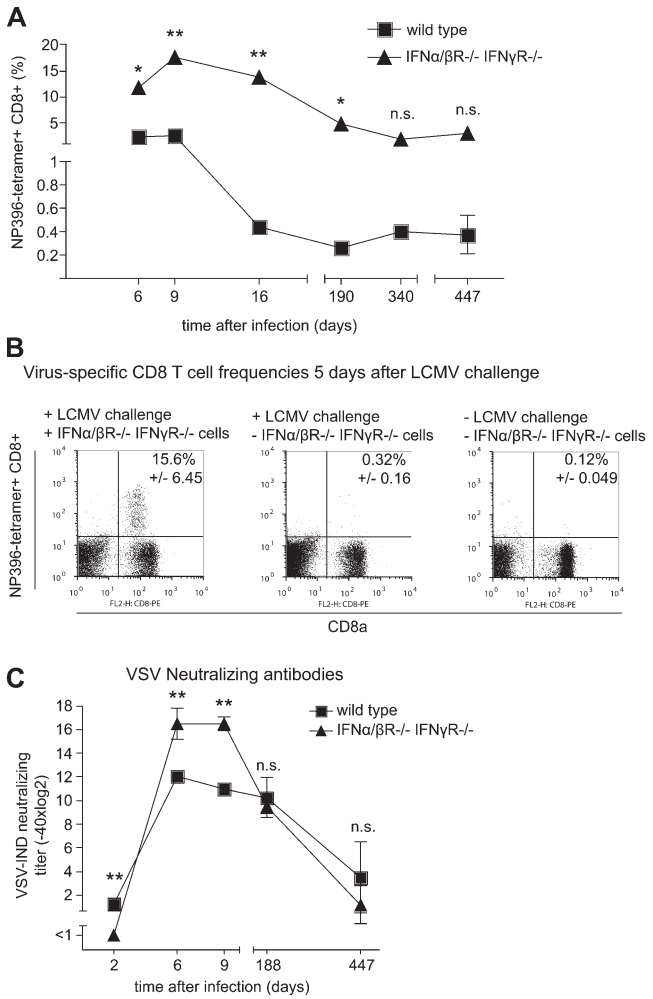

Fig. 3. Vigorous CD8$^+$ T cell and antibody responses in rLCMV/INDG-infected IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice. (A) IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ and wild-type mice were infected intravenously with LCMV/INDG. H2-D^b-restricted NP396-specific CD8$^+$ T cells (A and B) in peripheral blood were enumerated by FACS. (B) 10^7 splenocytes of IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ mice immunized 447 days previously with rLCMV/INDG were transferred into syngeneic recipient wild-type mice. NP396-specific CD8$^+$ T cells were measured 5 days after intracranial challenge with LCMV-ARM. (C) VSV-neutralizing serum antibody titers were assessed on the indicated time points. Symbols and bars represent the mean ± standard error of the mean of three to four mice per group.

underestimated, rather than overestimated, further supporting our conclusions that IFN type I is a main pathway of rLCMV/VSVG control *in vivo*. Lower IFN type I responses in wild-type mice to rLCMV/VSVG than to LCMV-ARM further highlight that IFN-mediated efficient containment of rLCMV/VSVG as compared with wild-type LCMV is not due to a differential IFN induction. Instead, it appears that the two viruses respond differentially to IFN type I and/or that the intrinsically different replicative potential combined with slower spread of rLCMV/VSVG (15–17) renders IFN type I more efficient.

rLCMV/VSVG elicited even higher frequencies of virus-specific CD8 T cells and stronger neutralizing antibody responses when inoculated into mice lacking IFN type I and II signaling. This unexpected finding contrasted with the documented type I IFN dependence of CD8 T cell responses to LCMV-ARM infection (34–37) and is likely due to higher viral antigen load. This is illustrated by the detection of 40-fold higher levels of rLCMV/INDG RNA levels on day 4 in IFNα/βR$^{-/-}$ IFNγR$^{-/-}$ compared with wild-type mice (Fig. 1D). However, further investigations are needed to dissect the underlying molecular mechanisms and examine alternative pathways such as IL-12 signaling—known to be suppressed by type I IFN during LCMV-ARM infection—which may bypass the type I IFN dependence of adaptive immune responses (42).

The discrepancy between considerable levels of RNemia and undetectable serum infectivity matches analogous findings in cell culture-grown virus preparations. rLCMV/INDG virus stocks consistently exhibited 10- to 100-fold higher viral RNA to PFU ratios than LCMV-ARM (data not shown). This difference can most likely be attributed to the lack of functional interactions between VSVG and the LCMV ribonucleoprotein (43). Incorporation of the foreign VSVG envelope protein into budding rLCMV/VSVG virions is presumably less efficient than for the GP of LCMV, representing one likely mechanism for rLCMV/VSVG attenuation (15). A molecular interplay between the LCMV-GP cytoplasmic domain, NP and the Z protein is involved in recruitment of the GP into budding LCMV virions, whereas particle formation occurs independently of GP and is driven by the Z protein (44). The 10- to 100-fold reduction in rLCMV/VSVG particle infectivity therefore seems to be a logical consequence of GP exchange, and the predominant production of non-infectious viral particles may explain why rLCMV/VSVG propagates more slowly than LCMV-ARM, both in cell culture and in infected mice (15–17). It remains currently unknown whether the differential dependence of the adaptive immune response on type I IFN to LCMV-ARM and rLCMV/VSVG is merely a consequence of slower replication of the latter virus or whether other factors such as altered host cell interference, differential cellular tropism and possibly yet other alterations of virus–host interactions contribute to this phenomenon in ways that may not necessarily be mutually exclusive.

The characteristics described here provide additional rationale (15) to test the safety and immunogenicity of envelope exchange-based arenavirus vaccines in non-human primates and eventually humans. To ensure potent cellular immune responses, the foreign envelope protein should mediate efficient entry of vaccine virus particles into human host cells, allowing for *in vivo* gene expression. In this respect, VSVG is a first-choice candidate since it is pantropic and efficiently fuses to all known human cell types, which also explains its wide use in gene therapy approaches (45). To assure sufficient gene expression, the vaccine virus should have the ability to replicate in human cells. Wild-type LCMV can cause severe disease in immunosuppressed transplant recipients (46) owing to unchecked viral replication. This suggests that a species barrier seems unlikely to prevent immunogenicity in humans while simultaneously emphasizing the need for stable attenuation.

Recently, we have also developed a replication-deficient vector systems based on LCMV (47), a platform aimed at inducing immunity to unrelated infectious agents or tumors.

In contrast, live-attenuated envelope exchange viruses are designed to induce cellular immunity to the homologous virus or to closely related arenaviruses. Replication competence of envelope exchange vaccines obviates the use of genetically engineered cell lines in the production process (47) and thus may represent an important practical advantage over replication-deficient vectors, both in terms of the ease of production and in production yields. Furthermore, replicating vaccine viruses may serve as potent booster formulations for arenavirus-specific immunity upon primary immunization with a replication-deficient vector. Thus, both approaches are complementary, and they each have their specific advantages and preferential areas of application.

In summary, this study has dissected the contributions of the individual arms of the adaptive and innate immune system to control of rLCMV/VSVG vaccine prototypes *in vivo*. Of particular relevance for future application in combating arenaviral hemorrhagic fevers, we have not observed clinically manifest disease when administering the vaccine to several mouse strains with severe and combined immunodeficiencies. Further, we found that rLCMV/VSVG induces potent and long-lasting memory T and B cell responses in the absence of IFN signaling, underscoring the excellent immunogenicity of this vaccine prototype even under conditions of partial immunodeficiency. These insights into the immunobiology of rLCMV/VSVG provide additional justification to further pursue GP exchange as a molecular strategy to engineer live-attenuated arenavirus vaccines, with the goal of inducing T cell-mediated immunity against LASV and related hemorrhagic fever viruses.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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